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Epstein-Barr Virus Reactivation Associated With Diminished Cell-Mediated Immunity in Antarctic Expeditioners

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Epstein-Barr virus (EBV) reactivation and cell-mediated immune (CMI) responses were followed in 16 Antarctic expeditioners during winter-over isolation at 2 Australian National Antarctic Research Expedition stations. Delayed-type hypersensitivity (DTH) skin testing was used as an indicator of the CMI response, that was evaluated 2 times before winter isolation and 3 times during isolation. At all 5 evaluation times, 8 or more of the 16 subjects had a diminished CMI response. Diminished DTH was observed on every test occasion in 4/16 subjects; only 2/16 subjects exhibited normal DTH responses for all 5 tests. A polymerase chain reaction (PCR) assay was used to detect EBV DNA in saliva specimens collected before, during, and after the winter isolation. EBV DNA was present in 17% (111/642) of the saliva specimens; all 16 subjects shed EBV in their saliva on at least 1 occasion. The probability of EBV shedding increased ($P = 0.013$) from 6% before or after winter isolation to 13% during the winter period. EBV appeared in saliva during the winter isolation more frequently ($P < 0.0005$) when DTH response was diminished than when DTH was normal. The findings indicate that the psychosocial, physical, and other stresses associated with working and living in physical isolation during the Antarctic winter result in diminished CMI and an accompanying increased reactivation and shedding of latent viruses. ***J. Med. Virol. 61: 235–240, 2000.*** Published 2000 Wiley-Liss, Inc.†

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INTRODUCTION

Epstein-Barr virus (EBV) is one of 8 currently recognized human herpesviruses and is capable of establishing a life-long infection of its human host. It infects an estimated 90–95% of the adult population worldwide and is the causative agent of infectious mononucleosis, Burkitt's lymphoma, nasopharyngeal carcinoma, and other rare lymphomas [Oxman, 1986; Rand et al., 1990; Lennette, 1991]. The establishment of viral latency and subsequent reactivation are not well understood, but factors such as emotional stress, trauma, sunlight, respiratory infections, fever, and specific changes in the immune response are known to increase the incidence and duration of reactivation and shedding of some latent viruses [Kasl et al., 1979; Glaser et al., 1985, 1995; Gosselin et al., 1992].

Latent viruses pose an important infectious disease risk to astronauts' health during space flight, and this risk almost certainly increases as the duration of space missions increases. Restricting preflight contact of the flight crews with high-risk populations reduces risks associated with many infectious agents [Taylor et al., 1997]. Risks associated with latent viruses, however, remain unabated by such precautions.

The immune system, specifically the cell-mediated immunity (CMI) component, typically limits EBV infections after reactivation and prevents further systemic disease [Tosato et al., 1984]. Decreased CMI response may lead to viral reactivation, resulting in asymptomatic viral shedding, localized infections, or disseminated infections. Previous studies demonstrated decreased CMI response during space flight [Taylor and Janney, 1992; Taylor, 1993].

The Australian Antarctic Division operates research stations on the Antarctic continent and on a sub-Antarctic island station year-round, and many science

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investigations have been conducted during their 50-year presence [Lugg, 1994]. These stations are among the most isolated on Earth and expose expeditioners to various types and levels of stress. Living on one of these stations is similar in many ways to living in space, and recently the Antarctic has been identified as a valuable ground-based analog to simulate some aspects of space flight [Ember, 1998]. The CMI response has been studied extensively at the Antarctic stations and been found to decrease during the winter-over isolation period [Williams et al., 1986; Muller et al., 1988, 1995a,b]. This study was undertaken to assess concurrently the CMI and the reactivation and shedding of EBV during the 8-month total physical isolation of an Antarctic winter-over expedition.

MATERIALS AND METHODS

Subjects

Sixteen subjects (14 males and 2 females) aged 26–56 years participated in this study at 2 Antarctic stations. Ten of these subjects were from the Davis station and 6 from the Mawson station. Both stations are inside the Antarctic Circle. All expeditioners selected for the winter isolation undergo a rigorous medical examination to ensure a healthy population for the 9 to 10 months of total physical isolation during the Antarctic winter. No physical contact with outside populations occurs during the winter isolation because of the harsh, remote environment. All human study protocols were approved by the Ethics Committee of the Australian Antarctic Division and the Institutional Review Board of the Johnson Space Center. All the subjects included in the present study were seropositive for EBV.

Saliva Samples

Saliva samples were collected upon arising between 1 and 3 times a week before, during, and after the isolation. A total of 642 saliva samples were collected, 359 from 10 subjects at Davis station and 283 from 6 subjects at Mawson station. Samples were stored at −70°C until the completion of the winter-over period, when they were placed in liquid nitrogen and returned by ship to the Australian Antarctic Division in Hobart, Tasmania, Australia. Subsequently, specimens were packed in dry ice and flown to the Johnson Space Center in Houston for analysis.

Sample Processing

Saliva specimens were concentrated by spinning at 8000 rpm (6726 × *g*) for 2 hr in a 100 KD filtration unit (Filtron Technology Corp., Northborough, MA) and extracted by a nonorganic extraction method (Qiagen Inc., Chatsworth, CA). Microcarrier gel (2 μl/ml) (Molecular Research Center Inc., Cincinnati, OH) was added to facilitate DNA recovery at the proteinase K digestion step (Boehringer Mannheim, Indianapolis, IN). DNA was resuspended in 50 μl of water (sterile nuclease-free biotechnology grade) (Amresco, Solon, OH). EBV DNA for control studies was obtained from Sigma Chemical Co. (St. Louis, MO). The following polymerase chain reaction (PCR) primers directed at the EBV polymerase accessory protein gene (BMRF1) were used: P1, 5′-3′ GTC CAA GAG CCA CCA CAC CTG (The Midland Certified Reagent Co., Midland, TX) and P2, 5′-3′ Biotin CCC AGA AGT ATA CGT GGT GAC GTAGA (Digene Diagnostics, Gaithersburg, MD). These primers were used at a concentration of 200 μM with 10 μM deoxyribonucleic acid triphosphates (Perkin-Elmer, Branchburg, NJ). PCR was optimized with buffer II (Perkin-Elmer, Foster City, CA) with 2.5 mM $MgCl_2$, using Perkin-Elmer GenAmp system 9600. DMSO (Sigma, St. Louis, MO) was added to a final concentration of 5%. AmpliTaq Gold (Perkin-Elmer, Foster City, CA) (2.5 units per 100 μl reaction mixture) was added, and 5 μl of the purified DNA was added to 20 μl of the reaction mixture. The cycle parameters were 95°C for 9 min, followed by 40 cycles of 94°C for 15 sec, 61°C for 15 sec, and 72°C for 15 sec, with a final extension step at 72°C for 5 min. PCR fragments were detected with the PCR Sharp System (Digene Diagnostics, Gaithersburg, MD) after 24 hr [Payne et al., 1999]. Positive and negative controls were used in the amplification by PCR and detection of EBV by Digene's Sharp Signal Detection system. Our laboratory verified that sensitivity of the Digene detection system approached 10 copies of DNA, as claimed by the manufacturer. Results of a control study, that included seronegative and seropositive samples, were compared with results from the test subjects. No cross-reactivity was observed between the EBV primers used and DNA from other herpesviruses (HSV type 1 and type 2, CMV, and HHV6).

Cell-Mediated Immunity Assessment

Delayed-type hypersensitivity (DTH) skin testing was used as an indication of cell-mediated immunity (CMI) [Shearer, 1999]. Subjects' DTH reactions were measured on 5 different occasions: 2 before the winter isolation and 3 during the isolation period. The CMI Multitest (Institut Mérieux, Lyon, France), with a disposable standardized intradermal antigen applicator, was loaded with 7 antigens and a glycerine control [Kniker et al., 1979; Muller et al., 1995a,b]. The antigens in this test were tetanus, diphtheria, *Streptococcus*, tuberculin, *Candida albicans*, *Trichophyton*, and *Proteus*. Reactions were recorded after 48 hr as the area of induration measured in millimeters with calipers; reactions were considered positive if the diameter of induration was ≥2 mm. Subject score was recorded as the total millimeters of induration from all 7 test antigens. The following criteria were used to assess CMI multi-test results [Williams et al., 1986; Muller et al., 1995a]: subjects with 1 or less positive antigen reaction, with a subject score of less than 5 mm, were designated anergic; subjects with subject scores of less than 10 mm were hypoergic; and subjects with subject scores of more than 10 mm were designated normal responders. All DTH testing was conducted by station physicians trained in the procedures.

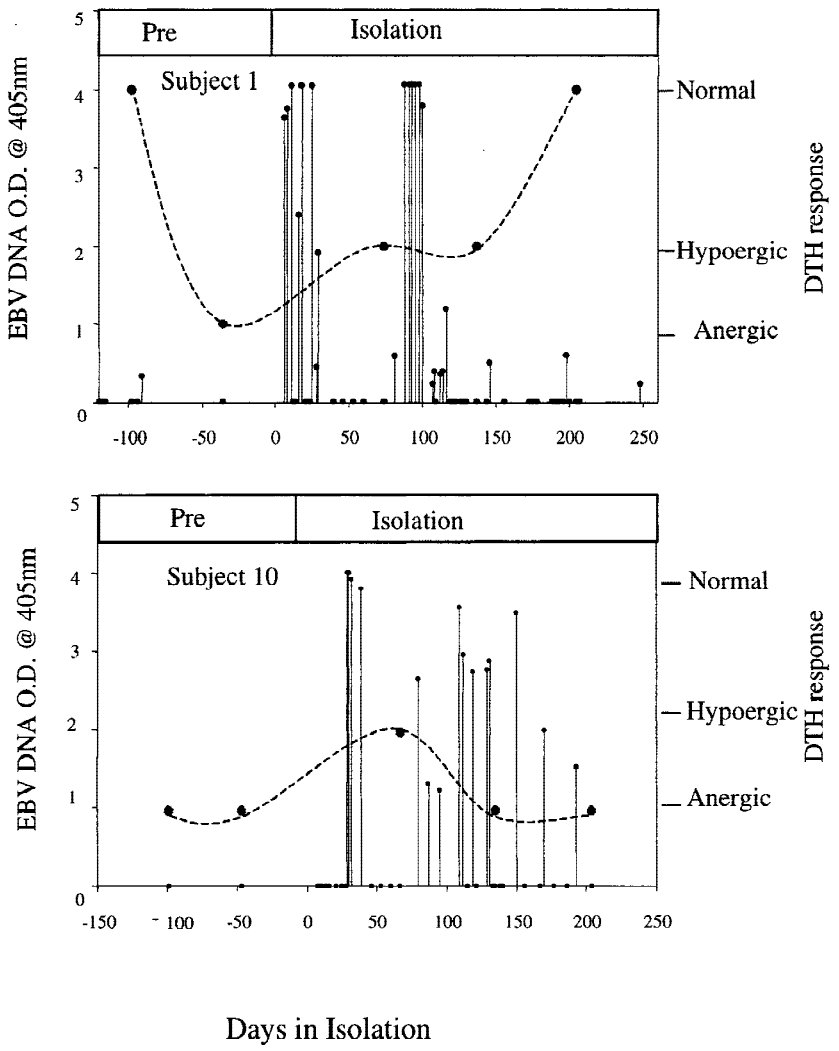

Fig. 1. Two subjects' viral shedding patterns as shown by the optical density of Epstein-Barr virus DNA at 405 nm and cell-mediated immunity (CMI) status before (Pre) and during winter isolation.

Statistical Analysis

A multilevel statistical model [Goldstein, 1995] with a binomial response variable was used to compare the incidence of viral shedding during the isolation and non-isolation periods. This model assumes random differences between subjects' probability of shedding are normally distributed on a logistic scale. To study the effect of CMI on the propensity for EBV shedding, we did not assume normally distributed errors at the subject level, but instead used the method of generalized estimating equations (GEE) [Zeger et al., 1988; McCullagh and Nelder, 1989]. The less presumptive GEE model was used because only 5 measurements of DTH were obtained per subject. Both methods take into account the repeated observations on subjects throughout the study period.

RESULTS

Sixteen subjects from 2 stations were studied during an Antarctic expedition to determine their EBV DNA shedding patterns and CMI status. Table I shows that

TABLE I. CMI Multitest Responses of 16 Subjects During the Pre-Isolation and Isolation (8-month Antarctic winter-over) Periods

	DTH response				
	Pre-isolation		Isolation		
Subject	Test 1	Test 2	Test 3	Test 4	Test 5
1	N	A	H	H	H
2	A	N	H	N	H
3	N	A	N	H	H
4	A	A	A	A	A
5	N	H	N	A	A
6	A	N	H	H	A
7	N	H	N	N	N
8	A	A	A	A	A
9	H	A	N	A	H
10	A	A	H	A	A
11	A	N	N	N	N
12	A	A	H	A	A
13	N	N	N	N	N
14	N	N	N	N	N
15	H	N	N	A	A
16	A	N	H	H	A

Subjects 1–6 were from Mawson station and subjects 7–16 from Davis station. N = normal; H = hypoergy; A = anergy.

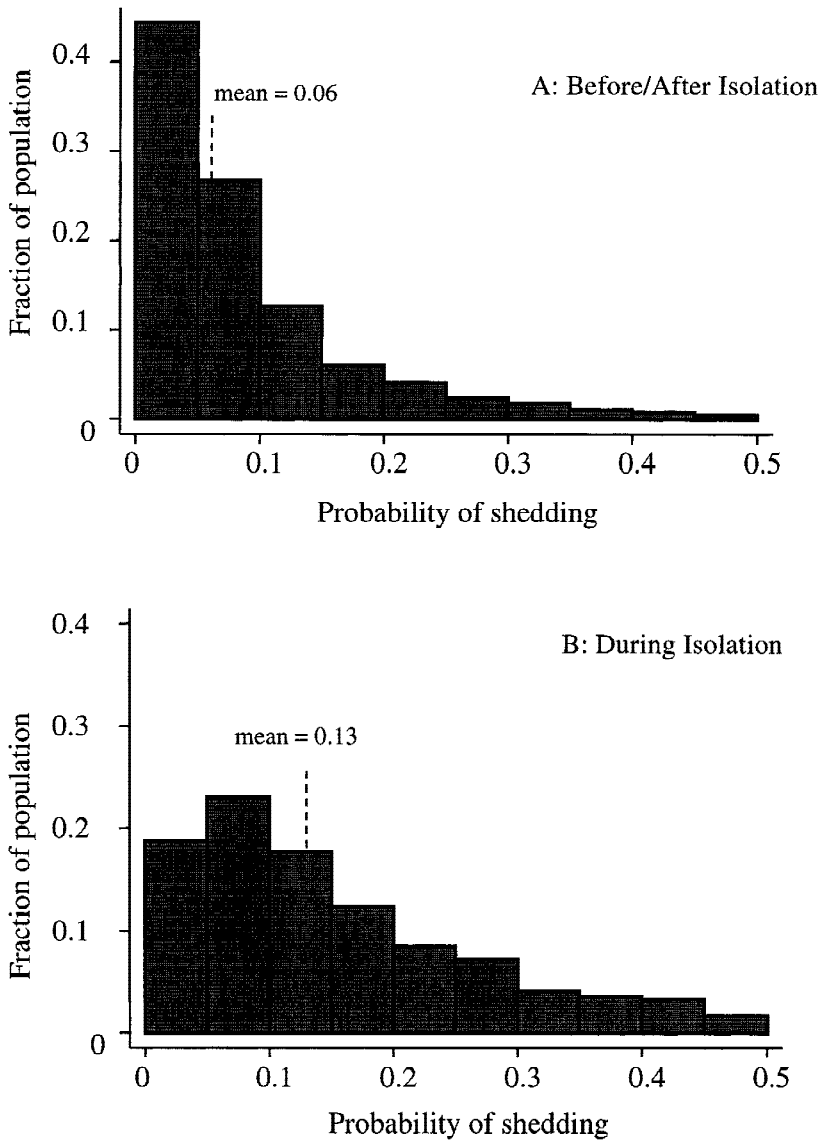

Fig. 2. EBV shedding probability before and after isolation (A) and during winter isolation (B) in Antarctica.

8 to 11 of the 16 subjects at any one of the 5 DTH test times exhibited diminished (either hypoergy or anergy) DTH responses. Only 2 of 16 subjects had a normal DTH response at all test times; 4 subjects had diminished DTH at all test times.

All 16 subjects shed EBV DNA during the expedition. EBV DNA was detected in 17% (111/642) of the samples collected from subjects before, during, and after the winter isolation period. As expected, the shedding frequency varied considerably among individuals. The shedding frequencies observed at the 2 stations, however, were not significantly different ($P = 0.168$). Salivary shedding frequencies varied from 2 to 39% among the expeditioners. Shedding patterns of 2 subjects with the highest shedding frequencies (39% and 38%) are shown in Figure 1. The DTH reaction of both subjects was diminished (hypoergic or anergic) at 2 or more test times during the winter isolation. Of the 10 DTH tests conducted on these 2 subjects during the study, 8 resulted in anergic or hypoergic reactions.

The presence of EBV DNA in the 16 expeditioners was significantly greater in saliva samples collected during winter isolation than before the isolation period. With a multilevel logistic model, the probability of EBV shedding was found to significantly increase ($P = 0.013$) from 6% before or after winter isolation to 13% during the winter period (Fig. 2).

Using a generalized linear model to account for random differences between subjects, we examined EBV DNA data during periods of diminished CMI (Fig. 3). During winter isolation, EBV DNA was shed more frequently when CMI was diminished (24%, 86/358 samples) than when CMI responses were normal (6%, 13/207). A similar trend was observed before and after the winter isolation, but the small amount of data collected before and after isolation was insufficient to es-

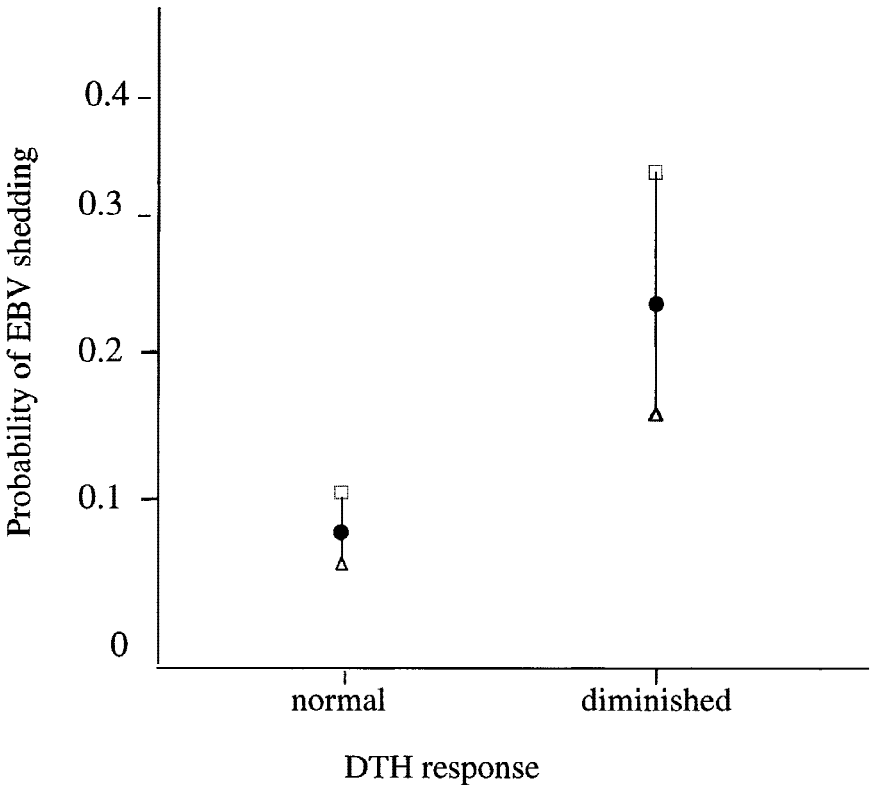

Fig. 3. EBV shedding and CMI response during isolation. Circles represent best estimates of probability of EBV shedding with the indicated CMI response. Upper and lower 90% confidence limits are indicated by squares and triangles respectively.

tablish a statistically significant correlation between EBV DNA shedding and diminished CMI.

DISCUSSION

This is the first report of increased salivary EBV shedding with diminished CMI response in Antarctic expeditioners. Investigators in the Australian National Antarctic Research Expeditions have previously shown immune changes in subjects at 3 Antarctic stations, including diminished CMI responses during the winter isolation [Williams et al., 1986; Muller et al., 1988, 1995a,b; Schmitt and Schaffar, 1993; Tingate et al., 1997]. Our observation of diminished CMI responses in over 50% of the subjects during winter isolation agrees with previous findings. For example, Muller et al. [1995a] reported 36% hypoergy in subjects in the Antarctic; Lugg (unpublished results) has reported levels up to 60% diminished CMI in wintering groups of Antarctic expeditioners. Hypoergy has been reported to be 10% in France, 5% in Australia, and 5-10% in USA [Kniker et al., 1979, 1984; Frazer et al., 1985]. Baseline DTH measurements (under low stress conditions in Australia) were not conducted in our study due to the subjects' unavailability during the desired baseline periods; however, Muller et al. [1995b] showed that the diminished DTH responses observed during a 56-day expedition in Antarctica returned to normal values after the subjects returned to Australia. Taylor [1993] reported similar DTH reductions in astronauts during short space flights; Konstantinova et al. [1993] found reductions in DTH responses during long space flights.

EBV DNA was found in saliva of astronauts participating in four 9- to 14-day flights aboard the space shuttle [Payne et al., 1999]. DTH was not measured on these missions. EBV DNA shedding, however, was monitored during 60- and 90-day closed-chamber studies and was found to occur more frequently during the in-chamber phase than before or after isolation [Mehta et al., 1998]. CMI status was measured (using DTH reaction) during the 90-day chamber study and was found to be diminished similar to those reported in the Antarctic studies and during space flight.

In the current study, the observed diminished DTH response and increased EBV DNA shedding in saliva may be the result of stress during the winter isolation. It is interesting that no clinical symptoms were attributable to the observed decreased cellular immunity and increased EBV DNA shedding during the winter-over isolation. Perhaps increased EBV shedding may serve as an early, asymptomatic indicator of decreased cellular immunity. Wood et al. [2000] have described various types of stress experienced during the Antarctic winter, and Glaser et al. [1995] have described the effects of various stressors on EBV reactivation. Meehan et al. [1992] described the effects of psychoneuroendocrine factors during space flight. Many of these effects are mediated through the hypothalamus-pituitary-adrenal axis. Future studies integrating

stress evaluations and measurements of stress hormones, immune functions, and latent virus reactivation are scheduled for an upcoming Antarctic expedition.

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